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### Stress, learning and hippocampal plasticity

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*Albino laboratory rat*

*The rat is probably the most commonly used laboratory animal. The albino, which has been developed by selection over the last seventy years from *Rattus Norvegicus*, differs in many important aspects of behaviour from its wild ancestor. It has been selected for tameness and differs from the wild rat in its behaviour towards man. There is a reduced tendency to flee from man, or to struggle or bite on being handled. Physically, the laboratory rat attains a lower body weight, and it has smaller adrenals and a smaller brain and spinal cord. It is also less resistant to cold as it seems to lack the ability to grow a thicker coat in cold conditions. Furthermore, under the controlled condition of the laboratory there is no particular breeding season. The albino rat is a traditional animal for behavioural research. Its ability to find its way to and through the branching passages of the burrow, enable it to find its way readily through a maze (modified from R.J. Olds and J.R. Olds).*

*R.J. Olds and J.R. Olds. A Colour Atlas of The Rat - dissection guide- Wolfe Publishing Ltd, 1979: ISBN 0723407320*

## Chapter 3

### **Exposure to Chronic Psychosocial Stress and Corticosterone in the Rat: Effects on Spatial Discrimination Learning and Hippocampal Protein Kinase C $\gamma$ Immunoreactivity**

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## Abstract

Previous reports demonstrated a striking increase of the immunoreactivity of the  $\gamma$ -isoform of Protein Kinase C (PKC  $\gamma$ -ir) in Ammon's Horn and Dentate Gyrus of rodent hippocampus after training in a spatial orientation task. In the present study we investigated how eight days of psychosocial stress affects the ability to learn a spatial discrimination task, and influences the training-enhanced PKC  $\gamma$ -ir in various regions of the hippocampus. Spatial discrimination learning and memory performance were assessed in a hole board in which the animals learned the pattern of 4 baited out of 16 holes. This study included four groups of adult male Wistar rats: (1) Naive animals; (2) animals Trained by 10 daily sessions with four holes baited in a fixed pattern; (3) Stressed animals exposed to eight days subordination stress and (4) Stressed animals that were exposed to the eight days social stress and subsequently Trained in the hole board. Learning performance of T and ST groups were calculated for reference- (RM) and working memory (WM). The acquisition of both RM and WM was significantly delayed in the stressed animals during the entire training period. With respect to cellular plasticity, the training experience in both non-stressed and stressed groups yielded enhanced PKC  $\gamma$ -ir in the CA1 and CA3 region of the posterior hippocampus but not in subfields of the anterior hippocampus. The stress experience itself significantly enhanced PKC  $\gamma$ -ir in the DG and CA3 pyramidal cells of the anterior hippocampus. In stressed animals that were subsequently trained, the PKC  $\gamma$ -ir was increased in the posterior CA1 region to the same level as found in non-stressed trained animals. Training after stress in these animals apparently abrogated the PKC  $\gamma$ -ir stress-response in the CA3 region. Secondly, we investigated whether the stress-induced alterations in learning and PKC  $\gamma$  could be attributed to the stress-evoked rise in plasma corticosterone levels. In these experiments rats were supplied with either a subcutaneous 50% corticosterone or a cholesterol control pellet and subsequently trained in the hole board. It was demonstrated that the corticosterone treatment did not significantly influence reference memory scores but slightly and temporarily affected working memory. When compared to naive animals, spatial discrimination learning enhanced PKC  $\gamma$ -ir selectively in posterior hippocampal CA1 and CA3 pyramidal neurons and their apical dendrites. The training-induced enhancement of PKC  $\gamma$ -ir in the CA1 region was similar in trained and corticosterone treated animals. The learning-induced PKC  $\gamma$ -ir response in the posterior CA3 area, however, was absent after corticosterone pre-treatment. In conclusion, our results demonstrate that prolonged psychosocial stress causes spatial learning deficits while the elevation of corticosterone levels to values as observed during such stress only mildly affects spatial memory performance. The spatial learning deficits following stress are only in part reflected in the redistribution of hippocampal PKC  $\gamma$ -ir following training.

## Introduction

The experience of stress triggers a complex neuro-endocrine cascade that leads among others to the release of glucocorticoids from the adrenal gland. Both stress and glucocorticoids exert striking influences on cognitive behaviors. Brief exposure to stress can enhance the acquisition of a classically-conditioned eyeblink response (Shors et al., 1992; Shors and Servatius, 1995), whereas long-term stress was shown to impair spatial discrimination in rats (Luine et al., 1994; Bodnoff et al., 1995). Evidence provides support for the view that cognitive deficits following prolonged stress are mediated by glucocorticoids. Long-term exposure of rats to high doses of corticosterone attenuates cognitive performance (Arbel et al., 1994; Bodnoff et al., 1995; Luine et al., 1993; Dachir et al., 1993), while removal of the adrenal gland and replacement of low levels of corticosterone prevents stress-induced cognitive deficits (Bodnoff et al., 1995). Moreover, stress and stress-induced levels of corticosterone impair long-term potentiation (LTP) and primed burst potentiation (PBP) in the hippocampus, two phenomena considered to represent cellular changes underlying mechanisms of learning and memory (Foy et al., 1987; Shors et al., 1989; Bennet et al., 1991; Diamond et al., 1990, 1992, 1994; Pavlides et al., 1993, 1995ab; Kerr et al., 1994). Several studies on the cellular and molecular basis of learning and memory point to a role for protein kinase C (PKC) in memory processing. PKC, a phospholipid and  $\text{Ca}^{2+}$ -dependent kinase is a key enzyme in signal transduction and neuronal plasticity. Changes in the distribution of PKC in the hippocampus, which has been implicated in many studies as a brain region pivotal to associative and spatial cognitive processes (McNaughton et al., 1986; Schmajuk et al., 1990; O'Keefe, 1979; Nadel 1991; Zola Morgan and Squire, 1990; O'Mara, 1995), have been associated with several forms of associative learning (Olds et al., 1989, 1990; Scharenberg et al., 1991; Van der Zee et al., 1992, 1995a; Beldhuis et al., 1992; Sunayashiki-Kusuzaki et al., 1993). In addition, PKC inhibitors and PKC activators like phorbol esters alter memory performance (Zhao et al., 1994; Paylor et al., 1991; Nogués et al., 1996), while mouse hippocampal PKC activity correlates positively with the ability to learn a spatial discrimination task (Wehner et al., 1990). Of the different isoforms of PKC present in the brain the  $\gamma$ -subtype of PKC was demonstrated to be the most abundant representative in the rat hippocampus (Saito et al., 1988; Huang et al., 1988) and which is associated with spatial

learning performance (Van der Zee et al., 1992; Van der Zee et al., 1995a; Beldhuis et al., 1992; Abeliovich et al., 1993). In the latter studies Van der Zee et al. (1992) and Beldhuis et al. (1992) demonstrated a striking increase of the immunoreactivity of the  $\gamma$ -isoform of Protein kinase C (PKC $\gamma$ -ir) in the pyramidal cell layer of the Cornu Ammonis 1 (CA1) and dentate gyrus (DG) of mouse and rat hippocampus after training in a spatial orientation task, which were partly explained by the stress component of the learning paradigm. The first aim of the present study was to investigate the impact of prolonged stress on reference- and working memory. Rather noxious stimuli or severe physical stressors have been used by most studies investigating the effects of stress on memory. The more naturally occurring stress is of psychological origin and has a profound impact on behavior, neurobiology, and physiology of rodents (Blanchard et al., 1993; Krugers et al., 1996). For that reason we investigated the effect of prolonged psychosocial stress - resulting of submission to a dominant male rat (Koolhaas et al., 1990; Krugers et al., 1996) - on cognitive performance. In addition we investigated whether stress-induced changes in spatial learning are accompanied by changes in the immunocytochemical redistribution of PKC $\gamma$  in the hippocampus following spatial discrimination learning. The second question approached was whether the stress-induced alterations in learning and PKC $\gamma$  could be attributed to the stress evoked rise in plasma corticosterone levels.

## Materials and Methods

### *Animals*

The experimental protocols were approved by the Committee on Animal Bio-Ethics of the University of Groningen. Male Wistar rats of 4 months of age, bred in our own facilities, were housed in groups of 6-7 animals per cage and kept on a 12 h lights on 12 h lights off cycle (7:00-19:00 dark). Food and water were available ad libitum.

### *Stress and corticosterone treatment*

Animals were exposed to eight days prolonged subordination stress by confrontation with a selected dominant male TMD S3 rat (Koolhaas et al., 1990; Krugers et al., 1996). The confrontation took place in a wooden cage (85 x 60 x 50 cm) which was permanently occupied by the dominant rat. The social interaction by definition resulted in display of submissive behavior by the

experimental animal. After submission the experimental rat was put into a small nestbox in the home cage of the dominant rat for the next eight days. The small nestbox was inaccessible to the dominant rat. In a pilot study, the impact of this social stress on plasma corticosteroid levels during the eight-day stress experience was investigated. Therefore, seven animals were provided with a permanent silicon catheter (0.95 mm OD, 0.50 mm ID) in the right atrium inserted via the right jugular vein (Steffens, 1969). The animals were allowed to recover one week from surgery. Subsequently two animals were housed individually (controls) and five animals were socially stressed. Blood samples of 0.45 ml were taken daily one hour after lights were switched off. Immediately after withdrawal the blood samples were transferred to chilled (0°C) centrifuge tubes containing 10  $\mu$ l heparin (500 IU/ml), centrifuged for 20 minutes at 3,500g and stored at -20 °C until further processing. Corticosterone was extracted from 75  $\mu$ l plasma using a liquid extraction method (Shimizu et al., 1983). Quantification of plasma corticosterone was performed by high pressure liquid chromatography (HPLC) in combination with ultraviolet detection. The absolute detection threshold for corticosterone in plasma was 8 ng/ml. In the second experiment we investigated the effect of corticosterone pellet implantation on spatial orientation in the hole board. Corticosterone (4-pregnene, 11 $\beta$ , -21-diol-3,20-dione; Sigma, St. Louis, Mo) pellets were formed by slowly heating corticosterone and cholesterol (5-cholestene-3 $\beta$ -ol; Sigma, St. Louis, Mo) in a 1:1 ratio. The 50% corticosterone pellets weighed approximately 100 mg each (see Meyer et al., 1979). The animals were anaesthetized with ether and the pellets implanted subcutaneously in the abdominal cavity at least 2 cm caudal to the incision. Whether these pellets produced transiently elevated circulating plasma corticosterone levels as observed during the eight days psychosocial stress was checked by a small control experiment. This revealed that implantation of a 50 % corticosterone pellet resulted in plasma corticosteroid levels ranging from  $25.5 \pm 4.3$   $\mu$ g/dl on the first day after implantation which then slowly decreased to  $11.7 \pm 1.7$   $\mu$ g/dl on day 8 (unpublished observations). Cholesterol pellets (100 mg) served as control. Naive (N) animals were kept in groups and served as blank controls, whereas animals from the T group received a cholesterol pellet.

### *Spatial orientation task*

The hole board task was used for spatial orientation learning (Oades and Isaacson, 1987; Van der Zee et al., 1992; Beldhuis et al., 1992). The setup

consisted of a ground-floor plate of 70 x 70 cm with four rows of four equidistant holes (14 cm apart, 3.5 cm diameter, 3 cm deep). Animals were habituated to the procedure by introducing them to the hole board and then spent two trials of three minutes each on 5 consecutive days in the test apparatus with food in all holes. Next, the habituation period was followed by a training period of 10 daily sessions. During these sessions the animals were trained to find four holes which were supplied with an accessible food pellet arranged in a fixed symmetrical pattern. Each hole contained a food pellet at the bottom covered by a replaceable, perforated false bottom. This way the rats were unable to discriminate between baited and non-baited holes by olfactory stimuli. The animals were trained during two trials per day each lasting three minutes. At the start of each trial, the animals were placed in a start box attached to one of the walls of the hole board. After 10 seconds, a guillotine door between the start box and the arena of the hole board was lifted allowing the rat to enter the arena. A visit was scored when the nose of the rat was placed in a hole. Revisits to baited holes and visits to non-baited holes were recorded as errors. The animals were removed from the hole board after either all four holes were visited or after the total testing time. Between the trials the floor of the start box and the hole board was cleaned with a wet and a dry cloth. Reference memory was defined as the [number of visits and revisits to the baited holes] divided by [the total number of visits to baited and non-baited holes]. Working memory was calculated as the ratio of the [number of food rewarded visits] to the [number of visits and revisits to the baited set of holes] (Beldhuis et al., 1992; Van der Zee et al., 1992).

### ***Stress, corticosterone, and spatial orientation***

The animals were divided into four groups to establish the effect of eight days social stress on spatial learning in the hole board and PKC $\gamma$ -ir levels in the hippocampus: Naive (N, n=5); Trained (T, n=7); Stressed (S, n=6); and Stressed/Trained animals (ST, n=6). Naive animals were group-housed and served as controls while animals from the Stress (S) group were exposed to the eight days prolonged subordination stress. The animals from the training group (T) were habituated and subsequently trained in the hole board. Animals from the ST group were subject to the subordination stress and underwent the habituation and training procedure after the stress exposure as described for the T group.

To investigate whether the stress-induced alterations in spatial orientation and PKC could be attributed to the stress-evoked rise in plasma corticosterone

levels the animals were divided into four groups; Naive (N, n=6), Trained (T, n=7), Corticosterone treated (C, n=5) and Corticosterone treated/Trained animals (CT, n=6). Eight days after pellet implantation the animals from the T group were habituated to the hole board for five days and subsequently trained for ten days to find the four baited out of 16 holes in the hole board. The animals from the C group received a corticosterone pellet. Animals from the CT group were supplied with a corticosterone pellet and eight days after surgery they underwent the habituation and training procedure as described for the T animals.

### ***Immunocytochemical procedure***

Either 24 hours after the last trial (for the animals trained in the hole board), after the last day of the stress procedure (S), or 9 days after corticosterone pellet implantation the animals were sacrificed together with naive animals. All rats were deeply anaesthetized with ether and transcardially perfused with 30 ml heparinized saline (15 ml/minute) followed by 300 ml fixative composed of 3% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The adrenal- and thymus glands were dissected and weighed. The brains were removed from the skull and cryoprotected by overnight storage in 30% sucrose in 0.1 M PB. Thereafter, 30  $\mu$ m coronal sections were cut on a cryostat microtome and sections were processed for detection of PKC $\gamma$ . The immunocytochemical staining was performed on free floating sections as described below, all steps being identical and run simultaneously for the animals per experiment. The tissue sections were preincubated for 15 minutes in 0.1 % H<sub>2</sub>O<sub>2</sub> in phosphate-buffered saline (PBS), subsequently rinsed in PBS, and immersed in 5% normal sheep serum (NSS) in PBS for 30 minutes to reduce background staining. Next, the sections were incubated with the first antibody (36G9, monoclonal mouse anti-PKC $\gamma$  IgG raised against purified bovine PKC diluted 1:1 in 0.01 M PBS, [Cazaubon et al., 1989, 1990]) overnight at 4 °C under gentle movement of the incubation medium. After the primary antibody incubation, sections were rinsed in PBS and again preincubated with 5% NSS for 30 minutes before exposure to biotinylated sheep anti-mouse IgG (Amersham, diluted 1:200 in PBS) for 2 hours at room temperature (RT). Thereafter, the sections were thoroughly rinsed in PBS and incubated in streptavidin-HRP (Zymed, diluted 1:200) in PBS for 2 hours at RT. Finally after subsequent rinsing in PBS and Tris buffer, the sections were processed by diaminobenzidine (DAB)-H<sub>2</sub>O<sub>2</sub> reaction (30 mg DAB and 0.01%

H<sub>2</sub>O<sub>2</sub>/100 ml Tris buffer), guided by a visual check.

### Measuring Optical Density

Sections of the animals of different groups were qualitatively analyzed while the experimentors were unaware of the origin of the sample. In addition, the optical density (OD) of PKC $\gamma$ -immunoreactivity was measured with an image analysis system (IBAS) to obtain semi-quantitative data on the distribution of PKC $\gamma$ . The OD was measured in the CA1 and CA3 pyramidal cell layer and in the DG molecular- and granular cell layer at the anterior (I.A. 5.7, according to Paxinos and Watson, 1982) and posterior (I.A. 4.2) levels. The OD values of the corpus callosum served as a measure for non-specific background staining. Specific staining was calculated by subtraction of the OD of the background from the total OD. Four hippocampi per animal per level were analyzed. Data per group were averaged and compared for relative differences between groups

### Statistics

All data were averaged and calculated as mean  $\pm$  SEM per group. The reference memory and working memory scores were statistically analyzed with analysis of variance (MANOVA) for repeated measures using the stress as between-subjects factor and the daily training sessions as within subjects factor followed by post-hoc analysis. The OD of the different groups were per experiment compared with the OD of the N group and statistically analyzed by analysis of variance. Significance level was  $P < 0.05$ .

## Results

### Body weight, thymus- and adrenal weight, and plasma corticosteroid levels

#### Stress experience

Changes in body weight during the eight days of social stress are presented in Fig. 1A. Stressed rats lost up to 7% of their body weight during the first three days of the stress experience. From day 4 the stressed animals gained again body weight but their absolute weight remained lower when compared to non-stressed animals. Statistical analysis revealed a significant effect of stress on body weight [ $F(1,9)=23.98$ ;  $P < 0.001$ ]. The social stress regime had also a profound effect on adrenal- and thymus weight (Table 1). It reduced

relative thymus weight and increased relative adrenal weight. No significant stress effects on adrenal or thymus weights were observed in trained and stressed/trained rats. The pilot experiment revealed that rats subjected to eight days of subordination stress underwent significant changes in plasma corticosteroid levels (Fig. 1B). During the first 2 days plasma corticosteroid levels of stressed animals were significantly elevated but then gradually declined to control levels.

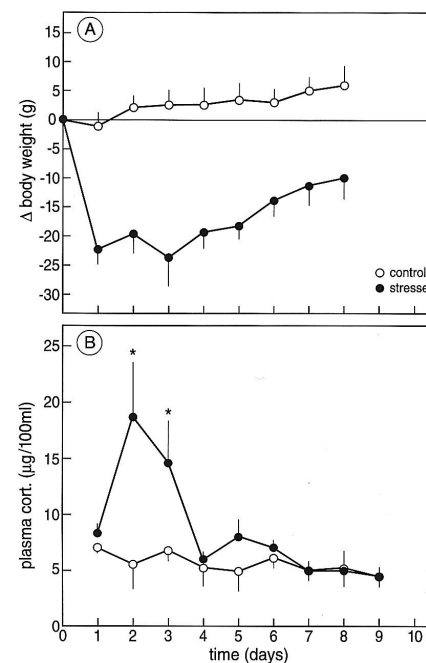


Figure 1. The effect of chronic stress on body weight gain (A) and plasma corticosteroid levels ( $\mu\text{g}/100\text{ml}$ ) (B). Values represent the mean  $\pm$  S.E.M. Asterisks represent significant differences from control group ( $P < 0.05$ ).

#### Corticosterone treatment

Rats implanted with a cholesterol pellet showed little weight-loss after the surgical procedure, and within 6 days their weight gain reached the preoperative value (Fig. 2). In contrast, rats implanted with a corticosterone pellet lost about 5% of their body weight in the first two days after the implantation and it took at least 11 days to regain this loss. Statistical analysis

Table 1. Chronic stress effect on thymus- and adrenal weight corrected for body weight.

Group	n	Thymus weight (mg/100g body weight)	Adrenal weight (mg/100g body weight)
Naive (N)	5	13.98 ± 1.00	5.24 ± 0.59
Stress (S)	6	5.95 ± 0.96*	8.45 ± 1.22*
Training (T)	7	13.07 ± 0.58	4.92 ± 0.53
Stress/Training (ST)	6	10.34 ± 1.22	6.00 ± 0.60

Values represent the mean ± SEM. Asterisks indicate significant differences from naive group.

by MANOVA showed a significant difference between the groups over the eight days period [ $F(2,6)=6.37$ ;  $P<0.001$ ]. The corticosterone pellet implantation reduced thymus weight while the adrenal weight was not significantly altered (Table 2). No effect of corticosterone on thymus weight was observed after subsequent training of the animals.

Table 2. Corticosterone effects on thymus- and adrenal weight corrected for body weight.

Group	n	Thymus Weight (mg/100g body weight)	Adrenal Weight (mg/100g body weight)
Naive (N)	6	10.02 ± 0.92	5.25 ± 0.43
Corticosterone (C)	5	7.55 ± 0.28*	4.44 ± 0.59
Training (T)	7	8.93 ± 0.60	10.02 ± 0.92
Corticosterone/Training (CT)	6	8.46 ± 0.79	4.98 ± 0.20

Values represent the mean ± SEM. Asterisks indicate significant differences from naive group.

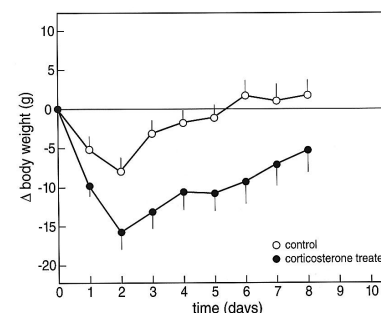


Figure 2. Changes in body weight during eight days of corticosterone treatment versus control rats. Body weight was significantly reduced over the entire period in the stressed animals when compared to control rats.

## Spatial orientation in the hole board

### Stress experience

Development of reference memory ratio (RM) and working memory ratio (WM) during training for non-stressed trained (T) and stressed/trained (ST) animals is presented in Fig. 3. The between-groups comparison indicated a highly significant reduction of RM in ST animals when compared to T animals over the entire period [ $F(1,11)=68.83$ ;  $P<0.001$ ] (Fig. 3A). Post-hoc analysis revealed significant main effects of stress on RM at days 5, 6, 7, 8 and 9 of the test period ( $P<0.05$ ). The overall conclusion will be that stress prior to hole board training significantly delayed the learning and acquisition of the long term memory cues. Moreover, also working memory was significantly reduced in the stressed animals (Fig 3B, [ $F(1,11)=4.51$ ;  $P<0.05$ ]). Significant main effects of stress on the WM component were observed at days 2 and 4 ( $p<0.05$ ).

### Corticosterone treatment

Development of RM (A) and WM (B) scores during the test period for both the T and CT rats are presented in Fig. 4. Both RM and WM improved during the course of the training period. The between-groups comparison for the entire training period indicated a similar progression in RM [ $F(1,11)=2.16$ ;  $P=0.154$ ] and WM [ $F(1,11)=0.42$ ;  $P=0.519$ ] for both the trained and corticosterone treated trained animals. However, the CT animals revealed a slower rise in the acquisition of WM in the initial phase of the learning test.

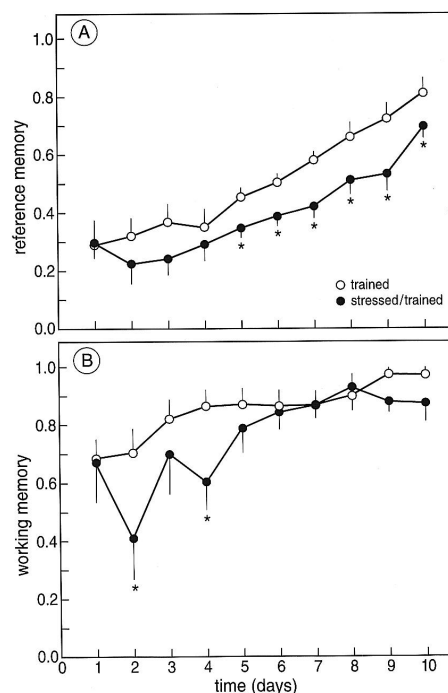


Figure 3. Development of reference memory ratio (A) and working memory ratio (B) of trained (T,  $n=7$ ) and stressed-trained animals (ST,  $n=6$ ). The between-groups measurements indicate a significantly impaired RM (A) and WM scores (B) for stressed animals over the entire period ( $P<0.05$ ). Analysis of variance indicates significant differences in both RM and WM at several days which are indicated by asterisks ( $*P<0.05$ ).

### PKC $\gamma$ -immunoreactivity Stress experience

The distribution of PKC $\gamma$ -immunoreactivity (PKC $\gamma$ -ir) in naive rat hippocampus was similar to that described previously (Van der Zee et al, 1992; Beldhuis et al., 1992; Saito et al., 1988). In short, PKC $\gamma$ -ir in naive untrained rats is very light for pyramidal cells in the CA1-3 region and granular cells in the DG. Faint PKC $\gamma$ -ir is present in apical dendritic extensions of these cell groups, while interneurons are devoid of PKC $\gamma$  staining. Hilar neurons, however, react positively to PKC $\gamma$  antibody staining. Habituation to the test chamber showed a tendency to increased PKC $\gamma$ -ir in DG granular neurons in both anterior and posterior hippocampus. This change did not reach levels of significance (data not shown).

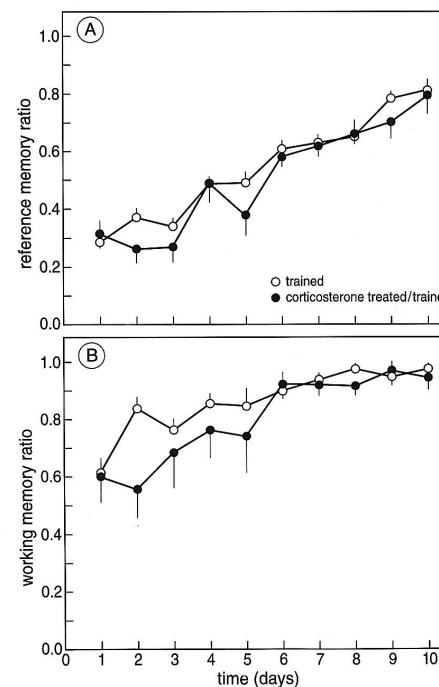


Figure 4. Development of reference memory ratio (A) and working memory ratio (B) of trained (T,  $n=7$ ) and corticosterone-treated trained animals (CT,  $n=6$ ). The between-groups measurements revealed neither significant differences in RM (A) nor in WM (B) ratio over the entire period ( $P<0.05$ ).

The eight days psychosocial stress regime appeared to induce specific and significant increases in PKC $\gamma$ -ir in the DG and CA3 of the anterior portions of the hippocampus. This stress-evoked PKC $\gamma$ -ir increase was particularly strong in anterior dentate granular cells and in their neurites in the dentate molecular layer. No such change was present at posterior CA3 and DG levels. These qualitative observations were substantiated by the semi-quantitative measurement of the optical density of PKC $\gamma$ -ir (Fig. 5 and 6). Training in the hole board did not affect PKC $\gamma$ -ir in the anterior hippocampus but training specifically enhanced PKC $\gamma$  staining in the posterior hippocampus, most notably in the pyramidal cell bodies and their apical dendrites in the stratum radiatum of the CA1 and CA3 region (Fig. 5, 6 and 7). Eight days of



stress did not influence the training enhanced PKC $\gamma$ -ir in the posterior CA1 region, which was similar as in trained animals without previous treatments (Fig. 7). In posterior CA3 neurons the preceding stress inhibited the training-induced increase in PKC $\gamma$ -ir (Fig. 5 and 6).

### Corticosterone treatment

The immunocytochemical distribution of PKC $\gamma$  in the hippocampus of naive untrained animals was similar as described above. In the anterior hippocampus non of the training conditions had an effect on PKC $\gamma$ -ir (Fig. 8). In the posterior hippocampus, however, training led to enhanced PKC $\gamma$ -ir in large areas of the CA1 and CA3 region. These changes were most prominent in the pyramidal cell bodies and their apical dendrites in the stratum radiatum (Fig. 9). While the effect of training on the enhanced PKC $\gamma$ -ir in posterior CA1 pyramidal cells was similar in control and corticosterone treated animals, it was abrogated by corticosterone treatment in the posterior CA3 pyramidal region (Fig. 9).

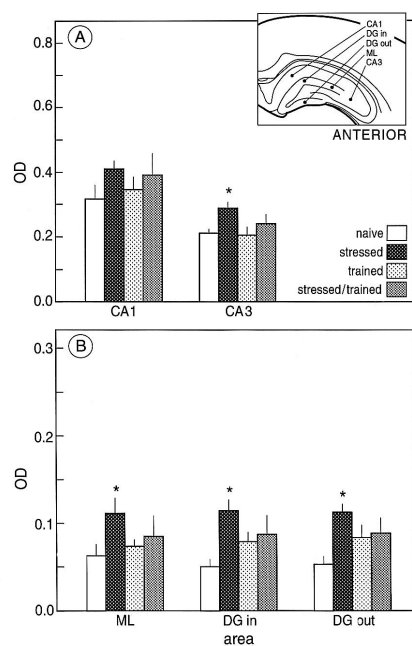


Figure 5. Optical density (OD) of PKC $\gamma$  immunoreactivity (PKC $\gamma$ -ir) (A) in the CA1 and CA3 pyramidal cell layer, and (B) in the dentate gyrus molecular layer (ML) and the inner (DGin) and outer (DGout) blade of the dentate gyrus granular cell layer in the anterior hippocampus (I.A. 5.7 according to Paxinos and Watson, 1982). Stress significantly increased PKC $\gamma$ -ir in the CA3 pyramidal cell layer (A) and dentate gyrus granular and molecular layer (B) when compared to naive animals (\* $P < 0.05$ ). Naive ( $n=5$ ); Trained ( $n=7$ ); Stressed ( $n=6$ ); Stress/Training ( $n=6$ ). The insert at the top indicates the selected regions for measuring the optical density.

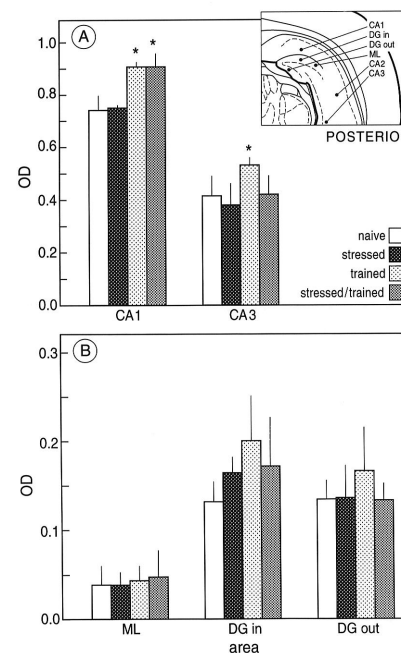


Figure 6. Optical density (OD) of PKC $\gamma$  immunoreactivity (PKC $\gamma$ -ir) (A) in the CA1 and CA3 pyramidal cell layer, and (B) in the dentate gyrus molecular layer (ML) and the inner (DGin) and outer (DGout) blade of the dentate gyrus granular cell layer in the posterior hippocampus (I.A. 4.2 according to Paxinos and Watson, 1982). Training enhanced PKC $\gamma$ -ir in the CA1 and CA3 pyramidal cell layers of both non-stressed (T) and stressed animals (ST). Naive ( $n=5$ ); Training ( $n=7$ ); Stressed ( $n=6$ ); Stress/Training ( $n=6$ ). The insert at the top indicates the selected regions for measuring the optical density.

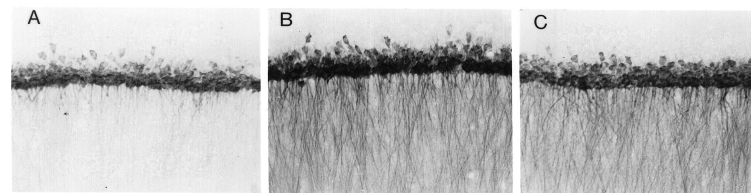


Figure 7. Photomicrographs of the CA1 region in the posterior hippocampus immunostained for PKC $\gamma$  in naive (A), trained (B) and stressed-trained (C) animals. Note that the pyramidal neurons and dendrites of both trained and stressed-trained animals are intensely stained for PKC $\gamma$  when compared to naive animals.

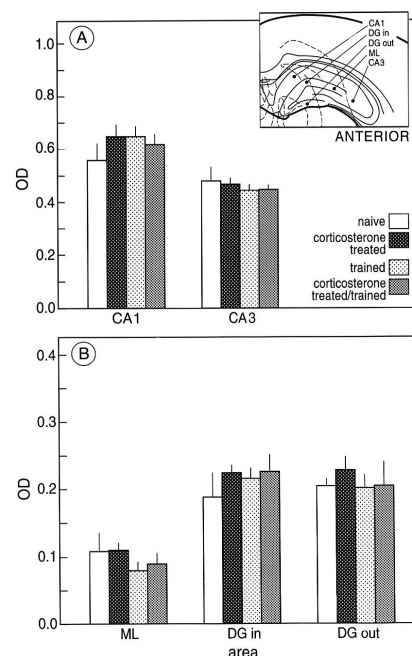


Figure 8. Optical density (OD) of PKC $\gamma$  immunoreactivity (PKC $\gamma$ -ir) (A) in the CA1 and CA3 pyramidal cell layer, and (B) in the dentate gyrus molecular layer (ML) and the inner (DGin) and outer (DGout) blade of the dentate gyrus granular cell layer in the anterior hippocampus (I.A. 5.7 according to Paxinos and Watson, 1982). No significant differences in the distribution of PKC $\gamma$  were observed between either of the groups. Naive ( $n=6$ ); Trained ( $n=7$ ); Corticosterone ( $n=5$ ); Corticosterone/Training ( $n=6$ ). The insert at the top indicates the selected regions for measuring the optical density.

## Discussion

The present study first shows that eight days of psychosocial stress impairs reference memory and working memory in a spatial orientation paradigm. Second, both hole board training and stress have an impact on the plasticity of PKC $\gamma$ -ir in the rat hippocampus. PKC $\gamma$ -ir in posterior hippocampal CA1 and CA3 pyramidal cells was enhanced by training, while stress specifically increased PKC $\gamma$ -ir in DG and CA3 at anterior hippocampal levels. Remarkably, stress prior to spatial learning did not affect the training enhanced PKC $\gamma$ -ir in the CA1 region of the hippocampus, but appeared to abrogate learning-induced PKC $\gamma$  changes in the CA3 region. Third, artificial elevation of plasma corticosterone levels to stress values did not affect reference

memory but affected the early phase of development of working memory. With respect to cellular plasticity the elevated corticosteroid levels did not affect the training-induced increase of PKC $\gamma$ -ir in the CA1 pyramidal cells but prevented the training enhanced PKC $\gamma$ -ir in the CA3 pyramidal cell layer.

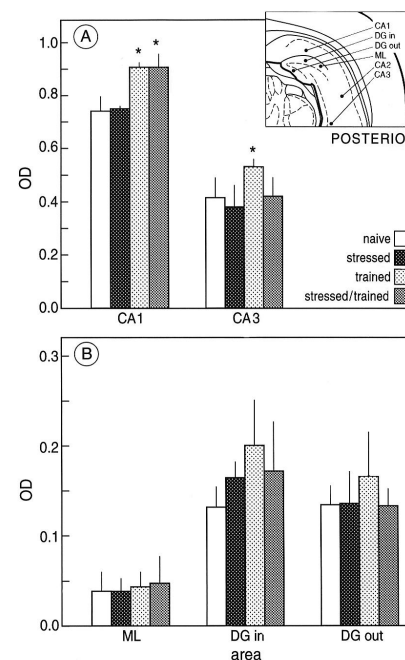


Figure 9. Optical density (OD) of PKC $\gamma$  immuno-reactivity (PKC $\gamma$ -ir) (A) in the CA1 and CA3 pyramidal cell layer, and (B) in the dentate gyrus molecular layer (ML) and the inner (DGin) and outer (DGout) blade of the dentate gyrus granular cell layer in the posterior hippocampus (I.A. 4.2 according to Paxinos and Watson, 1982). Training enhanced PKC $\gamma$ -ir in trained (T) and corticosterone treated/trained (CT) rats. Asterisks indicate statistically significant differences compared with the N group (\* $P<0.05$ ). Naive ( $n=6$ ); Trained ( $n=7$ ); Corticosterone ( $n=5$ ); Corticosterone/Training ( $n=6$ ). The insert at the top indicates the selected regions for measuring the optical density.

## Stress and memory

A couple of studies have demonstrated effects of prolonged stress on memory performance. Luine et al. (1994) reported impaired memory performance in an eight arm radial maze after three weeks of restraint stress, whereas Bodnoff et al. (1995) described learning deficits in a water maze after 6 months of social stress. In the present study we used eight days of psychosocial stress of submission to a dominant male rat to investigate the

impact of stress on learning in a hole board spatial discrimination task. This stress-paradigm caused an acute drop in body weight gain, transiently elevated basal corticosterone levels and altered adrenal- and thymus weight thereby corroborating the stressful character of the interaction between the experimental and dominant rat (Blanchard et al., 1993; De Goeij et al., 1992; Krugers et al., 1996). Our present results also reinforce the notion that prolonged stress causes learning deficits. Additionally, our data show that (a) social stress affects both reference and working memory, (b) a period of eight days of social stress is already sufficient to cause spatial learning deficits, and suggest that a relatively short stress period is followed by a long-term cognitive deficit: despite a 5 days gap between the termination of stress and the onset of training, the rats obviously failed to recover from the psychosocial stress experience.

#### ***Corticosterone and memory***

Several indirect lines of evidence suggest that corticosteroids are involved in the stress-induced reduction of memory performance (McEwen and Sapolsky, 1995; Arbel et al., 1994; Dachir et al., 1993). Direct evidence that corticosteroids may mediate spatial learning deficits after stress was presented by Bodnoff et al. (1995). They reported that mid-aged stressed animals, which were adrenalectomized and supplied with low levels of corticosterone failed to display cognitive impairments. The 50% corticosterone pellets in our study transiently elevated basal corticosterone levels to similar values as observed during the eight days social stress. However, this elevation had no effect on spatial discrimination learning. Reference memory scores were similar between control and corticosterone treated trained animals, and although corticosterone treatment slowed down the working memory acquisition rate in the early phase of the training period no significant differences over the entire period were observed. There may be some explanations why no cognitive effects of corticosterone were observed in the present study. First, corticosterone was reported to cause cognitive deficits when administered in supra-physiological dosages (Arbel et al., 1994; Bodnoff et al., 1995; Dachir et al., 1993) and during longer periods (Arbel et al., 1994; Bodnoff et al., 1995; Dachir et al., 1993; Luine et al., 1993) than in our study. Second, corticosterone appears to be more effective in attenuating spatial memory processes in mid-aged than in young adult rats used in the present experiments (Arbel et al., 1994; Bodnoff et al., 1995). Third, corticosterone may be responsible for stress effects on cognition during the actual elevation of this hormone level as suggested by a

few studies (e.g. Oitzl and de Kloet, 1992; Sandi and Rose, 1994ab). The long lasting action of stress as observed by us thus seems to involve other stress-related mechanisms (Bohus, 1994).

#### ***Stress, corticosterone and learning-induced alterations in PKC***

In the present study, we observed an increase in hippocampal PKC $\gamma$ -ir induced by spatial orientation in a hole board similar as reported previously using the same learning test (Beldhuis et al., 1992; Van der Zee et al., 1992, 1995a). Redistribution and changes in PKC $\gamma$  after learning are consistent with the observation that activation of PKC is necessary for proper spatial memory performance (Paylor et al., 1991; Wehner et al., 1990). The results on training-enhanced PKC $\gamma$ -ir are also in agreement with the reported changes in the intracellular distribution of PKC in the CA1 and CA3 region, which accompany associative memory storage within the hippocampus (Olds et al., 1989, 1990; Scharenberg et al., 1991; Van der Zee et al., 1992, 1995; Sunayashiki-Kusuzaki et al., 1993). Moreover, mouse hippocampal PKC activity correlates positively with the ability to learn a spatial discrimination task (Wehner et al., 1990). Besides our findings that confirm previous reports (Beldhuis et al., 1992; Van der Zee et al., 1992), the present results in addition show strong enhancement of PKC $\gamma$ -ir particularly in the posterior hippocampal CA1 and CA3 regions. It is at present not known whether altered PKC $\gamma$ -ir following training reflects changes in antigenicity or changes in molecular configuration. Previous observations by Van der Zee et al. (1992) suggest that the enhanced PKC $\gamma$ -ir following hole board training may result from conformational changes of PKC possibly as the result of activation of the protein. This is in agreement with the observation that the total amount of hippocampal PKC $\gamma$  is not altered after associative learning (Van der Zee et al., 1995b). In a recent survey study evidence is reported supporting the idea that PKC $\gamma$ -ir represents binding of the 36G9 antibody to exposed binding sites evoked by molecular unfolding after cellular activation (Van der Zee et al., 1997). Interestingly we observed that stress itself significantly increased PKC $\gamma$ -ir in the CA3 area and the DG molecular- and granular cell layer of the anterior hippocampus. Activation of PKC in the hippocampus after stress could possibly be explained by the stress-related release of glutamate (Moghaddam et al., 1993). Stimulation of metabotropic glutamate receptors activate PKC through hydrolysis of phosphatidyl inositol. In addition, activation of NMDA receptors by glutamate and the subsequent increase of intracellular Ca<sup>2+</sup> may

potentiate the  $\text{Ca}^{2+}$ -dependent activation of PKC $\gamma$ . The enhanced PKC $\gamma$ -ir following stress was not observed in stressed animals that were subsequently trained. However, it should be noted that in the stressed animals PKC $\gamma$ -ir was determined directly after the eight days of stress, whereas in the stressed animals that were subsequently trained the PKC $\gamma$ -ir was measured 16 days after termination of the stress experience. Therefore the blunted PKC $\gamma$  response of stressed/trained animals may be difficult to interpret due to the difference in survival time after the stress. It is tempting to speculate whether the regional differences in PKC $\gamma$ -ir after stress (anterior hippocampus) or training (posterior hippocampus) indicate differential contribution of these regions to sensory processing and/or memory (Amaral, 1993). Animals that were stressed or treated with corticosterone prior to training showed a similar distribution pattern of PKC $\gamma$ -ir in the posterior CA1 area when compared to trained animals. This suggests that eight days of subordination stress, which causes memory deficits, does not interfere with the regulation of PKC $\gamma$ -ir in CA1 following training in the hole board. It will be interesting to investigate whether stress interferes with PKC $\gamma$ -ir during the development of reference- and working memory. The observed increase in PKC $\gamma$ -ir in the posterior CA3 area of trained animals was not present in the stressed and corticosterone treated animals after training. It has been suggested that the neuronal mechanisms involved in impaired spatial learning following stress may be partly related to the stress- and corticosterone- induced dendritic shrinkage of apical dendrites of hippocampal CA3 pyramidal neurons (Woolley et al., 1990; Watanabe et al., 1992; Luine et al., 1994; Magarinos et al., 1995, McEwen and Sapolsky, 1995). The presently applied stress regime and corticosterone administration did not evoke degenerative changes in the hippocampus (Krugers et al., 1996) as assessed by silver impregnation methods. Nevertheless it remains to be investigated whether the eight days subordination stress causes dendritic atrophy. Besides, it is challenging to establish the relationship between altered memory performance, a decreased PKC response of posterior CA3 pyramidal neurons, and the cytology of CA3 apical dendrites. In this respect the link between PKC $\gamma$  and its effect on cytoskeletal protein dissociation might be an important observation (Tsuyama et al., 1986).

In conclusion, the present results demonstrate that prolonged psychosocial stress causes spatial learning deficits, whereas the elevation of corticosterone to stress cannot solely account for spatial memory impairment. The spatial learning deficits following stress are only in part reflected in the redistribution of hippocampal PKC $\gamma$  following training suggesting that PKC $\gamma$  is only

partially responsible for plasticity changes during and following memory formation. An interaction between corticosterone and other stress-related factors as demonstrated by a number of studies is likely to be involved in the presently observed stress-induced cognitive deficits (Bohus, 1994).

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